Application of the TRAP technique to lettuce (*Lactuca sativa* **L.**) genotyping[*]

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Summary

To demonstrate the applicability of the target region amplification polymorphism (TRAP) marker technique to lettuce genotyping, we fingerprinted 53 lettuce (*Lactuca sativa* L.) cultivars and six wild accessions (three from each of the two wild species, *L. saligna* L. and *L. serriola* L.). Seven hundred and sixty-nine fragments from 50 to 900 bp in length were amplified in 10 PCR reactions using 10 fixed primers in combination with four fluorescent labeled arbitrary primers. Three hundred and eighty-eight of these fragments were polymorphic among the 59 *Lactuca* entries and 107 fragments were polymorphic among the 53 lettuce cultivars and the six wild accessions; 251 fragments were present only in the wild species. These markers not only discriminated all cultivars, but also revealed the evolutionary relationship among the three species: *L. sativa*, the cultivated species, is more closely related to *L. serriola* than to *L. saligna*. Cluster analysis grouped the cultivars by horticultural types with a few exceptions. These results are consistent with previous findings using RFLP, AFLP, and SAMPL markers. The TRAP markers revealed significant differences in genetic variability among horticultural types, measured by the average genetic similarity among the cultivars of the same type. Within the sample set, the leaf type and butterhead types possessed relatively high genetic variability, the iceberg types had moderate variability and the romaine types had the lowest variability. The genetic behavior of TRAP markers was assessed with a mapping population of 45 recombinant inbred lines (RILs) derived from an interspecific cross between *L. serriola* and *L. sativa*. Almost all the markers segregated in the expected 1:1 Mendelian ratio and are being incorporated into the existing lettuce linkage maps. Our results indicate that the TRAP markers can provide a powerful technique for fingerprinting lettuce cultivars.

Introduction

Lettuce (*Lactuca sativa* L.) is grown worldwide as a vegetable crop. It is an increasingly popular vegetable in the U.S. because it is a basic ingredient in salads and is eaten more frequently than any other vegetable. According to the Economic Research Service of the USDA, an average American consumed 13.6 kg (30 pounds) of lettuce in 2000 (Glase et al., 2001).

Cultivated lettuce is classified mostly by vegetative morphology into five different types: iceberg or crisphead, romaine or cos, butterhead, stem, and leaf lettuces. The iceberg lettuce is the most important commercial type in the U.S. Its crisp textured leaves form large and dense heads with a white or creamy yellow interior. It gained popularity for its mild flavor, easy mass production, ease of packing for transportation, and also because a high salad volume can be made from a small head. The romaine or cos type of lettuce is important in Europe and is increasing its market share in the U.S. Its stiff, erect leaves form an elongated cylindrical head. In contrast, the butterhead lettuce produces a loose, soft head. It gets the name because its leaves have an oily or buttery feeling. The stem lettuce, also called asparagus lettuce, Chinese lettuce, or celtuce, is

a variety of lettuce grown mainly for its thick, edible stem. The leaf lettuce produces loose, frilly leaves of green or red color. For each type there are hundreds of cultivars available for commercial growers or home gardeners.

DNA-based molecular markers have become the tool of choice for crop genetic diversity assessment (Pujar et al., 1999; Hagen et al., 2002). Genetic variability is very important to a given crop because it is directly related to genetic vulnerability and the potential for genetic improvement. Molecular markers also have been used in cultivar identification (Hoffman et al., 2003; van Stallen et al., 2000), which helps the seed industry for the controlled propagation and marketing of valuable varieties. As a reliable and cost-effective alternative, DNA fingerprinting is replacing morphology as the traditional cultivar identification method. Morphological classification is costly, time and space-consuming, and sometimes difficult because many varieties have similar morphological characteristics, such as foliage shape and colors. Also, the expression of some of the traits can be influenced by environmental factors including light exposure, temperature, and soil fertility.

Molecular markers have previously been used in lettuce genomic studies, including construction of genetic maps (Kesseli et al., 1994; Jeuken et al., 2001), investigation of the diversity and origin of cultivated lettuce (Kesseli et al., 1991), examination of the genetic relationships among *Lactuca* species (Hill et al., 1996), and assessment of allelic diversity of selectively amplified microsatellite polymorphic loci (SAMPL) markers (Witsenboer et al., 1998). The Compositae Genome Initiative (http://cgpdb.ucdavis.edu/) has generated an enormous amount of DNA sequence data, especially EST (expressed sequence tag) information. Powerful bioinformatics tools have annotated the EST sequences as putative functional genes by homology searching against identified and characterized genes in the databases (http://cgpdb.ucdavis.edu/). The newly developed TRAP (Target Region Amplified Polymorphism) technique (Hu and Vick, 2003) takes advantage of the existence of the public EST databases and uses the sequences to design PCR primers against annotated EST sequences to detect polymorphic markers in order to link the EST sequences with phenotypes.

Identified first in *Drosophila* (McGinnis et al., 1984), homeobox genes exist throughout the plant and animal kingdoms and play an important role in the development of an organ (Gehring, 1987). The first homeobox member cloned from higher plants was the *kn1* (knotted 1) homeobox involved in leaf develop-

ment in corn. It was cloned by the transposon tagging approach (Vollbrecht et al., 1991). Later, *kn1*-like homeobox genes were documented in both monocot and dicot species. These genes encode DNA-binding regulatory proteins which recognize diverse DNA binding sites, act as transcription factors, and are organized into regulatory gene families (Scott et al., 1989). Thus, they are referred to as master control genes (Deshpande et al., 1998). In the present study, we applied the TRAP technique to fingerprint 59 lettuce accessions with 10 fixed primers designed against lettuce and sunflower EST sequences homologous to homeobox and disease resistance genes.

Materials and methods

Materials

Three sets of DNA samples derived from lettuce cultivars and wild relatives of lettuce were used. The first set consisted of 17 DNA samples isolated by the CTAB procedure. This set included 11 cultivars (*L. sativa*) of three horticultural types and three accessions from each of the two wild species (*L. serriola* L. and *L. saligna* L.), and was used in a preliminary fingerprinting experiment. The second set was comprised of DNA samples isolated with a DNeasy Plant Kit (QIAGEN, Valencia, CA). The samples were from the seedlings of 50 commercial lettuce cultivars in the UC lettuce cultivar collection. This set was for the main fingerprinting experiment. Eight cultivars were included in both sets as controls to check the reproducibility of the TRAP markers. The names and origin of the 59 entries (comprising 1 Latin-type, 14 butterhead, 14 iceberg, 13 leaf, and 11 romaine cultivars, plus six wild accessions) used in the current study are listed in Table 1. The third set of DNA samples was extracted with an in-house protocol (T. Wroblewski, unpublished) from a core mapping population of recombinant inbred lines (RILs) derived from an interspecific cross between *L. serriola* and *L. sativa*. This set was used to assess the genetic behavior of the TRAP markers.

Methods

The TRAP protocol standardized earlier (Hu & Vick, 2003) was followed.

DNA preparation

For the main fingerprinting project, three to five seeds from each cultivar were germinated on white blotter

Table 1. Lettuce cultivars and germplasm accessions included in the study

Cultivar name (or accession ID)	Horticultural type
A: *Lactuca sativa* (cultivated lettuce)	
Anuenue	Butterhead
Bibb	Butterhead
Butter crunch	Butterhead
Cobhamgreem	Butterhead
Dandie[b]	Butterhead
Diana[b]	Butterhead
Discovery	Butterhead
Kordaat[b]	Butterhead
Lednicky[b]	Butterhead
Mariska[b]	Butterhead
Mildura[a]	Butterhead
Ninja	Butterhead
Pybas Red Butter	Butterhead
Saffier	Butterhead
Solito[a]	Butterhead
Alpha[b]	Iceberg
Avoncrisp[b]	Iceberg
Bubba	Iceberg
Bullseye[a]	Iceberg
Calmar[b]	Iceberg
Desert Queen	Iceberg
El Dorado	Iceberg
Empire	Iceberg
Green Lake	Iceberg
Invader	Iceberg
Raiders	Iceberg
Salinas 88	Iceberg
Salinas 98G	Iceberg
Vanguard75	Iceberg
Argeles	Leaf
Colorado (red)	Leaf
Grand Rapids (green)	Leaf
Lolla Rosa (red)	Leaf
Natividad (red)	Leaf
Oak Leaf (green)	Leaf
Parella Red	Leaf
Prize head (red)	Leaf
Red Oak Leaf (red)	Leaf
Red Salad Bowl (red)	Leaf
Royal Oak Leaf (green)	Leaf
Ruby (red)	Leaf
Athena	Romaine
Clemente	Romaine
Darkland	Romaine

(*Continued*)

Table 1. (*Continued*)

Cultivar name (or accession ID)	Horticultural type
Greentowers	Romaine
Outback	Romaine
Parris Island	Romaine
Pinecrest	Romaine
Romo	Romaine
Tall Guzmaine	Romaine
Ultegra	Romaine
Valmaine	Romaine
Gallega	Latin
B. *L. saligna*	
CGN9311[a]	Wild
UC93US14[a]	Wild
PI-491204[a]	Wild
C. *L. serriola*	
W66336A[a]	Wild
94lact30-16[a]	Wild
94lact34-29[a]	Wild

[a]Entries used in the preliminary experiment.
[b]Cultivars were used in both preliminary and primary fingerprinting experiments.

paper soaked with water in plastic petri dishes for DNA extraction. Total DNA was isolated from 1-week-old whole seedlings using the DNeasy Plant Kit, following the manufacturer's instructions. The concentrations of DNA were determined with a DU7400 spectrophotometer (Beckman Coulter) and adjusted to 30 to 50 ng/μl for PCR reactions.

PCR primer design
Fixed primers. The fixed primers were designed against selected EST sequences in the Compositae Genome Project (http://cgpdb.ucdavis.edu/) for surveying genetic variability in Compositae species. In the current study, primers derived from six ESTs were used, including two lettuce and two sunflower ESTs homologous to the *Arabidopsis* homeobox genes, one sunflower EST homologous to a *Nicotiana tabacum* leucine-rich repeat (LRR) receptor-like protein kinase (Cho & Pai, 2000), and one sunflower unique gene (Table 2). The primers were designed by using the web-based PCR primer designing program "Primer 3" (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) (Rozen & Skaletsky, 2000) with the following parameters: primer optimum size, maximum size, and minimum size, 18 nt; primer

Table 2. Primers used in the current study

Name	Sequences (5′ to 3′)	Origin	Homologue[a]
Fixed primer			
QHA20I01a	CCGAGTTGGTATGCTTGT	Sunflower EST	gb|AAF66615.1| LRR receptor-like protein kinase (*Nicotiana tabacum*) and ref |NP_176789.1| receptor protein kinase (TMK1), putative; protein id: At1g66150.1 (*Arabidopsis thaliana*)
QHB14G14b	AATCTCAAGGACAAAAGG	Sunflower EST	No hits found
QHF6H21L	ACAGGAAAAGCCTGTCAC	Sunflower EST	ref|NP_181138.1| BEL1-like homeobox 1 protein (BLH1); protein id: At2g35940.1, (*Arabidopsis thaliana*)
QHF6H21R	CTGCTGCTGTTGAAGTTG	Sunflower EST	Same as above
QHG17L13L	TGGCTGTTTGAACACTTT	Sunflower EST	ref|NP_195405.1| BEL1-like homeobox 2 protein (BLH2); protein id: At4g36870.1, (*Arabidopsis thaliana*)
QHG17L13R	TGTTCATGTTCCTTGCAT	Sunflower EST	Same as above
QGA7H07L	AAGGATTCGGACAAACAT	Lettuce EST	ref|NP_181138.1| (NM_129153) BEL1-like homeobox 1 protein (BLH1); protein id: At2g35940.1, (*Arabidopsis thaliana*)
QGA7H07R	CACCATTGGCTTCCATAG	Lettuce EST	Same as above
GGB9J18L	TGGACTTCAACCAAGACA	Lettuce EST	gb|AAM63933.1| (AY086888) homeobox gene 13 protein (*Arabidopsis thaliana*)
GGB9J18R	TCTGTTGGCTCCCTATTT	Lettuce EST	Same as above
Arbitrary primer			
Ga3-800	TCATCTCAAACCATCTACAC		Arbitrary sequence
Ga5-800	GGAACCAAACACATGAAGA		Arbitrary sequence
Sa12-700	TTCTAGGTAATCCAACAACA		Arbitrary sequence
Sa4-700	TTACCTTGGTCATACAACATT		Arbitrary sequence

[a]By BLAST search.

Table 3. Primer combinations used in the current study

Name	Primer combinations
Lac01	QHF6H21L and Sa12-700 (IRD700) and Ga5-800 (IRD800)
Lac02	QHF6H21R and Sa12-700 (IRD700) and Ga5-800 (IRD800)
Lac03	QHG17L13L and Sa12-700 (IRD700) and Ga5-800 (IRD800)
Lac04	QHG17L13R and Sa12-700 (IRD700) and Ga5-800 (IRD800)
Lac05	QGA7H07L and Sa4-700 (IRD700) and Ga3-800 (IRD800)
Lac06	QGA7H07R and Sa4-700 (IRD700) and Ga3-800 (IRD800)
Lac07	GGB9J18L and Sa4-700 (IRD700) and Ga3-800 (IRD800)
Lac08	GGB9J18R and Sa4-700 (IRD700) and Ga3-800 (IRD800)
Lac09	QHB14G14b and Sa4-700 (IRD700) and Ga5-800 (IRD800)
Lac10	QHA20I01a and Sa4-700 (IRD700) and Ga5-800 (IRD800)

optimum T_{m}, maximum T_{m}, and minimum T_{m} to 53 °C, 55 °C, and 50 °C, respectively; and GC content between 40 and 60%.

Arbitrary primers. The sequence information for the four arbitrary primers used in the current study were provided by G. Li and C. Quiros (Li & Quiros, 2001 and personal communication). The primer combinations of the 10 PCR reactions are listed in Table 3. Each of the arbitrary primers contains three parts: (1) the selective nucleotides, 3 to 4 nts at the 3′ end, (2) the AT or GC rich "core" regions, 4 to 6 nts in the middle for annealing with an exon or an intron of a gene, and (3) the filler sequences which make the 5′ end, following the general principles of PCR primer designing such as the avoidance of self-complementarity, the maintenance of proper GC content (40 to 60%) for proper melting temperature of primers, and the retention of their correct internal stability. The arbitrary primers were 5′ end-labeled with IR dye 700 or IR dye 800 for auto-detection. The arbitrary primers rather than the EST-derived gene specific primers were labeled so that

the more expensive primers could be used in multiple assays.

PCR amplifications

PCR was conducted with a final reaction volume of 15 μl in 96-well microtiter plates in a GenAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following components: 2 μl of the 30 to 50 ng/μl DNA sample, 1.5 μl of 10× reaction buffer, 1.5 μl of 25 mM MgCl$_2$, 1 μl of 5 mM dNTPs, 0.3 pmol each of 700- and 800-IR dye labeled arbitrary primers, 10 pmol of the fixed primer, and 1.5 units of *Taq* DNA polymerase (QIAGEN, Valencia, CA). The PCR was performed by initially denaturing template DNA at 94 °C for 2 min; then 5 cycles at 94 °C for 45 s, 35 °C for 45 s and 72°C for 1 min; followed by 35 cycles at 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min; then a final extension step at 72 °C for 7 min.

Electrophoresis of amplified products

Upon completing the PCR cycles, 7 μl of 5× loading dye (containing 0.313 M Tris-HCl pH 6.8 at 25 °C, 10% SDS, 0.05% bromophenol blue, and 50% glycerol) was added to the reaction mixture. A 1-μl aliquot was loaded onto a 6.5% polyacrylamide sequencing gel in a Li-Cor Global DNA Sequencer with an 8-channel pipette. The sequencing gel was prepared using protocols recommended by the manufacturer (Li-Cor Biosciences). Electrophoresis was conducted at 1500 V for 3.5 h and the images were collected by the SAGA software (Li-Cor Biosciences).

Scoring and analyzing the amplified fragments

The polymorphic fragments were visually scored from the printed images. The scoring codes were 1 for present, 0 for absent, and 9 for missing. The data was analyzed with NTSYSpc, Numerical Taxonomy and Multivariate Analysis System version 2.11 (Exeter Software, Setauket, NY).

The Dice's coefficient (Dice, 1945) was used to calculate the pairwise genetic similarity (GS = $2a/(2a + b + c)$) matrices using the SIMQUAL procedure in the NTSYSpc software. The GS matrices were then used to construct the dendrogram with the UPGMA (unweighted pair-group method using arithmetic averages) algorithm (Sneath and Sokal, 1973), employing the SAHN (Sequential, Agglomerative, Hierarchical, and Nested) clustering procedure (Sneath and Sokal, 1973), and the principal coordinate (PCO) plot, using principal component analysis procedures such as

DCENTER (double center), EIGEN (eigenvectors) and MDSCALE (multiple dimension scaling).

Results and discussion

Reproducibility and genetic behavior of TRAP markers in lettuce

A small scale preliminary experiment was conducted to test the TRAP protocol (Hu and Vick, 2003) in lettuce. DNA samples of 17 accessions including 11 cultivars and three accessions of each of the two wild species were subject to TRAP amplification. The first six sets of PCR reactions that were run with the primers designed against the homeobox gene EST sequences produced over 200 polymorphic fragments. These markers not only differentiated all the cultivars but also were consistent with the previously identified evolutionary relationship among the three species (Kesseli & Michelmore, 1986; Kesseli et al., 1991; Hill et al., 1996; Witsenboer et al., 1998). These preliminary results suggested that the TRAP technique is reliable for lettuce fingerprinting.

The main fingerprinting experiment used 50 lettuce cultivars (Table 1). Ten sets of PCR with 10 fixed primers and four arbitrary primers in the combinations listed in Tables 2 and 3 were run with the three sets of DNA samples described in the section on "Materials." Since two arbitrary primers, each labeled with a different infrared dye (IR-700 and IR-800), were used in one PCR reaction with the fixed primer, 2 images were obtained. Although the three sets of DNA samples were prepared with different procedures in different laboratories and the PCRs were run independently, the same primer combination generated the same amplification profile (Figure 1), suggesting that the TRAP markers are highly reproducible. The high reproducibility was confirmed by the fingerprinting scores on the eight cultivars in the two independent experiments. Only 17 cases of different scores were observed between the two experiments: four for cultivar 'Alpha', one for 'Avoncrisp', three for 'Calmar', zero for 'Dandie', one for 'Diana', one for 'Kordaat', six for 'Lednicky' and one for 'Mariska'. On average, there were 2.1 incidents per cultivar. Taking into account that there were 107 polymorphic markers scored, the repeatability was approximately 98%.

DNA samples from a permanent mapping population containing 45 recombinant inbred lines (RILs) derived from an interspecific cross between *L. serriola*
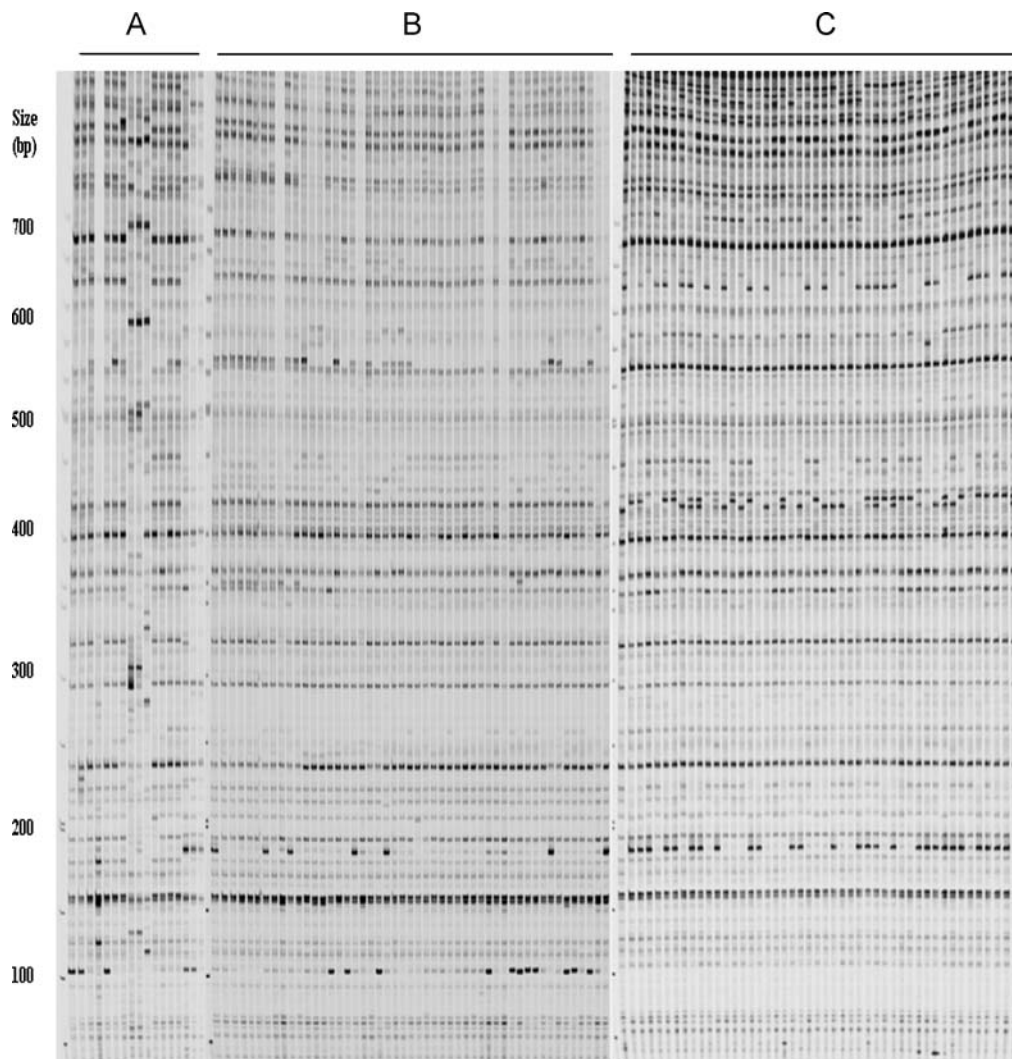
Figure 1. TRAP marker patterns (image from the primer combination Lac09-800) from three sets of lettuce DNA samples prepared with different laboratory procedures: (A) 17 lettuce entries in the pilot experiment; (B) 50 lettuce entries in the primary fingerprinting experiment; and (C) a segregating population of 45 RILs (Lanes 4 to 48 from left), the parental lines (Lanes 1 and 2 from the left), and the synthetic F1 hybrid (Lane 3). The fragment size standards were from Li-Cor and were sized from 50 to 700 bp.

and *L. sativa* were used to study the genetic segregation of the TRAP markers. Since the same primer combinations amplified the same fragments from this population as from the 50 cultivars and the wild relatives, markers with the same size can be traced to the two parental lines which were used in the fingerprinting experiment (Figure 1). Almost all the markers segregated in the expected 1:1 Mendelian ratio in the mapping population and initial mapping analysis with Map-Maker software indicated that the polymorphic markers were well spread across the genome with a few clusters, each with 2 to 5 tightly linked markers. Thus, the

TRAP markers are typical genetic markers. As shown in Figure 1, TRAP revealed a high level of polymorphism in this population; 10 PCRs amplified about 250 polymorphic markers. These markers are being incorporated into the existing lettuce linkage maps by the UC Davis group.

Genetic variability within and among cultivars of different types

The current genetic variability of cultivated lettuce is the result of factors intrinsic to the species

(self-pollination), but also is influenced by human factors (e.g. artificial selection, isolation, and interspecific crossing). Morphologically, the 53 lettuce cultivars belonged to four different types. The marker data collected enabled us to analyze the genetic variability within and among these types using the pairwise GS values calculated by the NTSYSpc software. Because pairwise GS values are the estimations of the proportion of markers that are identical in the two cultivars being compared, the higher the GS value, the lower the genetic variability between the two cultivars.

The GS matrix contains the pairwise GS values for all possible pairs among the 53 cultivars. They ranged from 26.1% to 99.0% and the distribution was nearly normal. For comparison of the GS among cultivars of the same type, six entries of the wild species and cultivar 'Gallega' were excluded because the latter is a single entry of the Latin type. The differences of average GSs among the four types were significant. The leaf type and butterhead type possessed relatively high genetic variability with average GS of 58.3% and 60.1%, respectively. There are four leaf-type cultivars which are closely related with GS values greater than 90%. The iceberg type had moderate variability with an average GS of 70.3%, while the romaine type had the lowest variability at only 82.9%. There are two sub-clusters within the romaine type containing five and six cultivars each (Figure 2). The GS values among the cultivars in the same sub-cluster are more than 90% but between the cultivars in different sub-clusters are less than 80%. The 14 iceberg cultivars were also clustered in two sub-groups, but the GS values among the cultivars within the group are substantially higher than those with the romaine type. This trend is well visualized by the two-dimensional PCO plot (Figure 3A) in which the cultivars of both romaine and iceberg types were plotted in the restricted areas, while the cultivars of leaf and butterhead types were more spread out. However, the detected differences of genetic variability within and among different types may not reflect the overall genetic diversity of the cultivated lettuce since only a limited number of cultivars were sampled for each type.

Phylogenetic relationship among the three species revealed by TRAP markers

A total of 388 fragments were scored among all 59 entries. Of these fragments, 221 and 70 were unique to *L. saligna* and *L. serriola*, respectively. Only 107 fragments were polymorphic among the 53 cultivated lettuce entries. Among these 107 markers, only 28 (26.2%) were confined within the cultivated types, 31 (29.0%) and 28 (26.2%) could be traced to *L. serriola* and *L. saligna*, respectively, and 20 (18.7%) were contributed by both wild species. These markers discriminated all cultivars tested.

The dendrogram (Figure 2), constructed from 388 polymorphic markers and 59 lettuce entries using the Dice similarity coefficient and UPGMA analysis, clearly reveals the phylogenetic relationship among the three species. *L. saligna* is more distantly related to the cultivated species *L. sativa* than is *L. serriola*. This relationship is better visualized by the 3-D plot that resulted from principal coordinate analysis based on genetic similarity matrices (Figure 3b). This was in accordance with the previous finding with isozyme, RFLP, and other DNA-based markers (Kesseli and Michelmore, 1986; Kesseli et al., 1991; Hill et al., 1996; Witsenboer et al., 1998).

All 14 iceberg-type, 12 leaf-type, and 12 romaine-type cultivars were grouped together in separated clusters in the dendrogram (Figure 2). For the butterhead-type cultivars, 11 formed a major cluster, but three cultivars were exceptions. According to the lettuce cultivar database (UC Davis *Compositae* Database), Cultivar 'Bibb' was the first American gourmet lettuce bred during the middle 1800s in Kentucky and is sometimes called Limestone lettuce. Cultivar 'Buttercrunch' was introduced by Dr. G. Raleigh at Cornell in 1963. It is somewhat like 'Bibb' but has large compact yellow-white hearts, is slow to bolt, and has a long-lasting firm crunchy texture. The grouping of 'Bibb' and 'Buttercrunch' may reflect common ancestry; they do have some morphological similarity. In the dendrogram, these two are closer to the old Latin-type cultivar 'Gallega', suggesting that they might have been selected from cultivars of the Latin type. Another cultivar, 'Anuenue', which resides in the iceberg cluster, formed a separate pair with 'Avoncrisp'. 'Anuenue' was developed in Hawaii and is classified as a semi-head variety. It has a smooth dark green "buttery" leaf and will form a larger head when conditions are suitable (Sakuoka et al., 2000). The molecular data suggest that there is genetic relatedness between 'Anuenue' and iceberg-type cultivars.

Gene flows among different types

Although lettuce is a self-pollinated species, there are no crossing barriers among different cultivar types, and
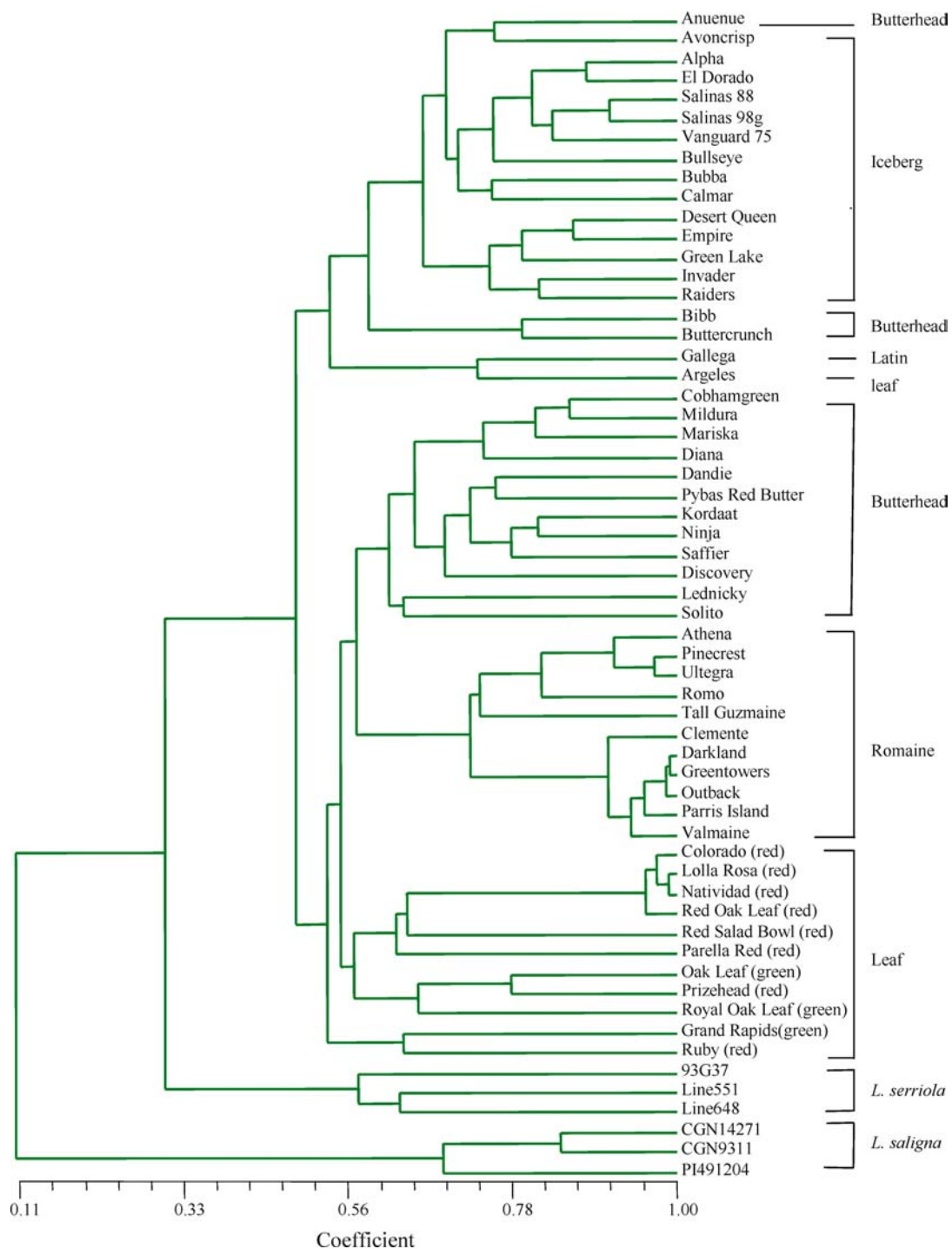
Figure 2. Dendrogram of percentage similarity among 59 entries of lettuce cultivars and their wild relatives based on the Dice similarity coefficient and UPGMA analysis.
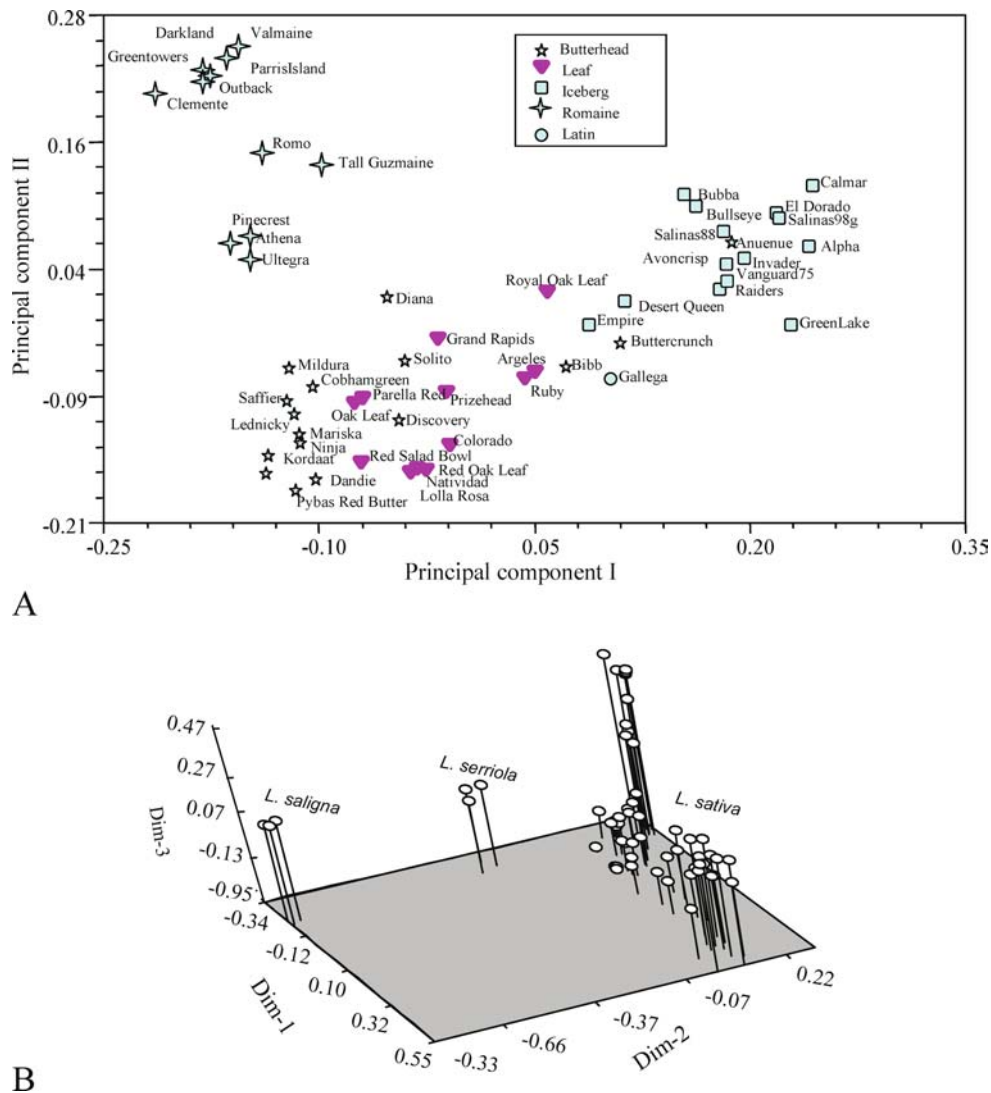
Figure 3. The principal coordinate analysis (PCO) plots of the 59 lettuce entries based on polymorphic TRAP markers. (a) plot of 53 lettuce cultivars based on 107 markers. Cultivars of different horticultural types are plotted in clusters with a few exceptions. The first two principal components account for about 31.6% of the total variation (18.6% for PC I and 13.0% for PC II, respectively); (b) 3-D plot of the 59 lettuce entries based on 388 markers revealed clearly the evolutionary relationship among the 3 species: *L. saligna* is more distantly related to the cultivated species *L. sativa* than is *L. serriola*.

between the cultivated lettuce and the two wild species used in this study. In breeding programs, many crosses have been made among different types and between species. The pedigrees of most of the entries are vague due to the confidentiality issue. Therefore, it is difficult to trace the gene flow by traditional pedigree analysis. The marker data can reflect the gene flows among the cultivars and between species. Among the 107 polymorphic markers (alleles) scored among the 4 types of cultivars, 28 can be traced in both wild species; 31 and 20 markers originated from *L. serriola* and *L. saligna*,

respectively, and 28 were confined within the cultivated species *L. sativa*. This is consistent with the relationship between *L. sativa* and *L. serriola* being closer than that between *L. sativa* and *L. saligna*. The former pair shares 59 (55%) common alleles while the latter shares only 48 (45%) common alleles.

Only a few type-specific markers were observed. The best example of this is marker Lac05801 (the first polymorphic marker on the IRD-800 image from primer combination Lac05), a 75-bp fragment which is present in each of the 11 romaine-type cultivars and

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absent from the rest of the 42 cultivars. This fragment is present in three *L. saligna* accessions but absent in *L. serriola*. Other markers include Marker Lac04803, which appeared only in 2 leaf-type cultivars: marker Lac10804, which was found in 4 butterhead-type cultivars, and markers Lac05706 and marker Lac10802, which were detected only in the butterhead cultivar 'Discovery'. It is known that 'Discovery' was developed from an interspecific cross between *L. sativa* and *L. saligna* and the latter contributed the lettuce downy mildew resistance. These two markers could be amplified from the introgressed chromosome segment since they could be traced to the donor species, *L. saligna*, and none of the other 52 cultivars possess these two fragments. Only 59 (55%) of the 107 alleles were shared by all four cultivar types since null alleles (absent in all the cultivars of a given type) have been observed for 48 (45%) of the 107 alleles.

The advantage of TRAP

Our results indicate that the TRAP markers can provide a powerful technique for fingerprinting lettuce cultivars. There are several advantages of TRAP over other DNA marker techniques. First, it combines the favorable features of the RAPD (easy to perform) and the AFLP (highly informative) methods. Second, TRAP explores the bioinformatics tools to design primers against known sequences of putative genes, while RAPD and AFLP are generated by random anonymous sequences. To ensure the amplification of the targeted EST sequence from which the fixed primer is designed against, the TRAP method uses unequal amounts of fixed and arbitrary primers in the PCR reactions, i.e. the amount of the fixed primer is 30-fold more than the arbitrary primer. In TRAP, each primer combination amplified 30 to 50 fragments and it is difficult to demonstrate which amplified fragments are related to the targeted gene (EST). However, it seems reasonable to assume that most of the fragments result from mismatching between the primers and the target sequences due to the low annealing temperature (35 °C) that was used during the first five cycles of the amplification. A few fragments should be related to the targeted sequence due to a perfect match with the fixed primer. The following Southern dot blot hybridization (Sambrook et al., 1989) experiment was carried out to examine the homology of the TRAP markers with the targeted EST sequences from which the fixed primers were designed. Two primers were designed from a lettuce EST sequence (M1625L and M1625R) and used in two

separate TRAP reactions as the fixed primer in combination with two arbitrary primers. TRAP amplified products (M1625R+arbitrary and M1625L+arbitrary) were cloned into pCRII vector with the TA cloning kit (Invitrogen, Carlsbad, CA). A set of 864 (2 × 384 + 96) random clones were transferred onto a nylon membrane and hybridized with a 300-bp fragment generated by amplifying lettuce genomic DNA using primers M1625R and M1625L. Washes were carried out at high stringency and an image was generated with a Typhoon Imaging System (Amersham, Piscataway, NJ). Twenty-one clones showed a hybridization signal (one very strong, eight strong and 12 weak). Taking into account that the nine clones showing strong hybridization signal have homology and were amplified by the fixed primer annealing to the targeted sequence in the TRAP reaction, we calculate that approximately 1% of the cloned TRAP fragments are from the targeted EST sequences. It is necessary to discuss the fact that the percentage of positive hybridization (9/864) doesn't correspond to the number of bands in the gel since there is the possibility of highly abundant bands not related to the target sequence being cloned preferentially. Therefore, the percentage of the EST related fragments in the gel could be higher than 1%. This result supported the aforementioned assumption. Another piece of supporting evidence for this assumption came from the genetic mapping of telomere associated sequence (TAS) markers to the linkage group ends using the TRAP technique when the fixed primer was designed against the conserved higher plant telomere sequence. Three TAS markers were mapped at linkage group ends in the mapping population. The conserved telomere sequence-derived fixed primer generated TAS markers mapped at the ends of linkage groups in sunflower (Hu et al., 2005) and in wheat (Liu et al., 2005). We observed that the fixed primers play an important role in generating the amplification profiles because the same arbitrary primer produced totally different amplification profiles when combined with different fixed primers. Since each TRAP reaction can amplify a large number of high quality markers, the TRAP technique can be utilized efficiently for plant genome mapping, gene tagging, and cultivar fingerprinting.

Acknowledgments

References

Cho, H.S. & H.S. Pai, 2000. Cloning and characterization of ntTMK1 gene encoding a TMK1-homologous receptor-like kinase in tobacco. Mol Cells 10: 317–24.

Deshpande, A.D., W. Ramakrishna, G.P. Mulay, V.S. Gupta & P.K. Ranjekar, 1998. Evolutionary and polymorphic organization of the knotted1 homeobox in cereals. Theor Appl Genet 97: 135–140.

Dice, L.R., 1945. Measures of the amount of ecologic association between species. Ecology 26: 297–302.

Gehring, W.J., 1987. Homeoboxes in the study of development. Science 236: 1245–1252.

Glase, L., G. Lucier & G. Thompson, 2001. Lettuce: in and out of the bag. Agricultural Outlook/April 2001. Website: http://www.ers.usda.gov/publications/AgOutlook/April2001/AO280d.pdf.

Hagen, L., B. Khadari, P. Lambert & J.M. Audergon, 2002. Genetic diversity in apricot revealed by AFLP markers: Species and cultivar comparisons. Theor Appl Genet 105: 298–305.

Hill, M., H. Witsenboer, M. Zabeau, P. Vos, R. Kesseli & R.W. Michelmore, 1996. AFLP fingerprinting as a tool for studying genetic relationships in *Lactuca* spp. Theor Appl Genet 93: 1202–1210.

Hoffman, D., A. Hang, S. Larson & B. Jones, 2003. Conversion of an RAPD marker to an STS marker for barley variety identification. Plant Mol Biol Reporter 21: 81–91.

Hu, J. & B.A. Vick, 2003. TRAP (target region amplification polymorphism), a novel marker technique for plant genotyping. Plant Mol Biol Reporter 21: 289–294.

Hu, J., J. Chen, A. Berville & BA. Vick, 2005. Defining sunflower linkage group ends with the conserved telomere sequence-derived TRAP markers. Plant Genome XIII. January 15–19, San Diego, CA.

Jeuken, M., R. van Wijk, J. Peleman & P. Lindhout, 2001. An integrated interspecific AFLP map of lettuce (*Lactuca*) based on two *L. sativa* × *L. saligna* F2 populations. Theor Appl Genet 103: 638–647.

Kesseli, R.V. & R.W. Michelmore, 1986. Genetic variation and phylogenies detected from isozyme markers in species of *Lactuca*. J. Hered. 77: 324–331.

Kesseli, R.V., I. Paran & R.W. Michelmore, 1994. Analysis of a detailed genetic linkage map of *Lactuca sativa* (lettuce) constructed from RFLP and RAPD markers. Genetics 136: 1435–1446.

Kesseli, R.V., O.E. Ochoa & R.W. Michelmore, 1991. Variation at RFLP loci in Lactuca spp. and origin of cultivated lettuce. Genome 34: 430–436.

Landry, P.A. & F.J. Lapointe, 1996. RAPD problems in phylogenetics. Zoologica Scripta 25: 283–290.

Li, G. & C.F. Quiros, 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. Theor Appl Genet 103: 455–461.

Liu, Z., J.A. Anderson, J. Hu, T.L. Friesen, J.B. Rasmussen & J.D. Faris, 2005. A wheat intervarietal linkage map based on microsatellite and target region amplified polymorphism markers and its utility for detecting quantitative trait loci. Theor Appl Genet (in press).

McGinnis, W., R.L. Garber, J. Wirz, A. Kuroiwa & W.J. Gehring, 1984. A homologous protein-coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. Cell 37: 403–408.

Pujar, S., S.A. Tamhankar, V.S. Rao, V.S. Gupta, S. Naik & P.K. Ranjekar, 1999. Arbitrarily primed-PCR based diversity assessment reflects hierarchical groupings of Indian tetraploid wheat genotypes. Theor Appl Genet 99: 868–876.

Rozen, S. & H.J. Skaletsky, 2000. Primer3 on the WWW for general users and for biologist programmers. In: S. Krawetz & S. Misener (Eds.) Bioinformatics Methods and Protocols: Methods in Molecular Biology, pp. 365–386. Humana Press, Totowa, NJ.

Sakuoka, R., R.T. Hamasaki, R. Shimabuku & A. Arakaki, 2000. Lettuce for the home garden. CTAHR's Home Garden Vegetable Series. Website: http: //www2.ctahr.hawaii.edu/oc/freepubs/pdf/HGV-2.pdf.

Sambrook, J., E.F. Fritsch & T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual, 2nd edn. , Cold Spring Harbor Laboratory, New York.

Scott, M.P., J.W. Tamkun & G.W. Hartzell, 1989. The structure and function of the homeodomain. BBA Reviews on Cancer 989: 25–48.

Sneath, P.H.A. & R.R. Sokal, 1973. Numerical Taxonomy: The Principal and Practice of Numerical Classification. W. H. Freeman and Company, San Francisco.

UC Davis Compositae Database: Website: http: //compositdb.ucdavis.edu/.

van Stallen, N., V. Noten, M. Demeulemeester & M. de Proft, 2000. Identification of commercial chicory cultivars for hydroponic forcing and their phenetic relationships revealed by random amplified polymorphic DNAs and amplified fragment length polymorphisms. Plant Breed 119: 265–270.

Vollbrecht, E., B. Veit, N. Sinha & S. Hake, 1991. The developmental gene *Knotted-1* is a member of a maize homeobox gene family. Nature 350: 241–243.

Witsenboer, H., J. Vogel & R.W. Michelmore, 1998. Identification, genetic localization and allelic diversity of selectively amplified microsatellite polymorphic loci (SAMPL) in lettuce and wild relatives (*Lactuca* spp.). Genome 40: 923–936.